Salmonella Species and *Campylobacter jejuni* Cecal Colonization Model in Broilers

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ABSTRACT *Salmonella* and *Campylobacter* are of concern to the poultry industry because of the continuing association of poultry-borne transmission of these diseases to humans. Live, mature bird interventions can be demonstrated only by comparing colonization in nontreated groups of control birds with treated bird groups. This study attempted to create a reproducible broiler chicken colonization model. When chicks were challenged 2 d posthatch with both *Salmonella* and *Campylobacter*, cecal colonization was achieved. By 4 wk posthatch, *Salmonella* counts per gram of cecal content diminished to very low or nondetectable levels. *Campylobacter* counts remained high throughout the test period. To achieve the goal of creating a mature bird *Salmonella* intestinal colonization model, oral treatment of 10 to 25 mg of vancomycin was given to 4-wk-old broilers, and 3 h later a composite of 3 *Salmonella* isolates were gavaged into the chickens. Birds were sampled 1 and 2 wk later. The data indicated that colonization was achieved at levels of 10^{6-7} cfu g^{-1} of cecal materials (at wk 5) and $>10^2$ to 10^4 cfu g^{-1} of cecal materials (at wk 6).

Key words: *Salmonella*, *Campylobacter*, colonization, mature broiler

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INTRODUCTION

Poultry-borne transmission of *Salmonella* spp. and *Campylobacter jejuni* remain a public health concern worldwide and within the United States (Balsley, 2006; Cardinale et al., 2003). Poultry products are often considered to be an important infectious route for humans. Despite substantial regulatory and industrial efforts to control bacterial pathogens, the frequency of contaminated carcasses has trended upward (USDA, 2007). Most efforts to control the organism within the United States have primarily been dedicated to the processing of broiler flocks. There is an unfortunate inability for the top poultry processors to control *Salmonella* contamination consistently (ElAmin, 2006; Food and Water Watch, 2006). Because consumer demands for fresh chilled poultry remain high, processing measures, short of carcass irradiation, appear to require interventions during broiler production to enable control of *Salmonella* and *Campylobacter*.

Hatchery-borne broiler transmission appears to be an important source for *Salmonella* spp. Broiler companies routinely monitor hatcheries in response to detection of *Salmonella* and may immunize breeder flocks against those contaminating serotypes. This approach provides a measure of protection against the hatchery-borne isolates until the bacterium begins to mutate and alter antigenic structure, when immunity is lost. In addition, new serotypes emanating from environmental sources may infect either the breeders or the broilers, and new, short-lived immunization or antibiotic therapeutic interventions may be enacted. Antibiotic treatment of chickens may result in undesirable resistance developing in contaminating *Salmonella* (Berrang et al., 2006). Using a greater level of biosecurity appears to have benefits for pathogen control in some Northern European production facilities (Heyndrickx et al., 2002). Such biosecurity measures provide protection for only a few additional production days before the entire flock becomes contaminated with *Campylobacter* spp., and such interventions can be quite difficult to enforce uniformly (Jacobs-Reitsma et al., 1995; Berndtson et al., 1996). Additionally, such high levels of biosecurity in intensive production facilities within the United States and in a large portion of poultry-producing countries are not uniform and are not practiced effectively.

To study how nonimmunization alternative interventions might provide on-farm pathogen control, a reliable model for both young and mature broiler colonization was needed. This manuscript provides the stepwise progression in developing such a model.

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MATERIALS AND METHODS

Bacterial Strains

A composite of 3 *Salmonella* spp. isolates (*Salmonella* Montivideo 845, *S.* California 706, *Salmonella* Heidelberg 130) were used in this study. The strains are from the Poultry Microbiological Safety Research Unit culture collection and were selected based on 2 criteria. First, the isolates were nalidixic acid resistant for ease in direct plate enumeration, and second, they were known to be good broiler colonizers based on previous studies. Individual *Salmonella* spp. isolates were grown overnight (16 to 18 h) at 37°C on *Brucella* agar plates, growth was resuspended in PBS (pH 7.2), absorbance was determined at 540 nm, and equal numbers of each isolate were dispersed into suspensions containing the desired cfu per milligram. *Campylobacter jejuni* isolates BL-1 and AL-22 (Stern et al., 2001) were grown for 24 h at 42°C under a microaerobic atmosphere on Brucella agar plates, and subsequently, growth was resuspended in PBS at the desired logarithmic dilutions of cfu per milliliter, and suspensions were combined to provide a single *Salmonella* spp. and *C. jejuni* challenge per chick.

Bacterial Challenges of 2-d-old Broilers

Approval for the following animal experiments was provided by the Institutional Animal Care and Use Committee ("Control of *Campylobacter* in Poultry Production"). A total of 400 broiler chicks were used in the first portion of the study. Mucosal competitive-exclusion (**MCE**) flora were prepared as described previously and consisted of an undefined healthy adult microflora, with no *Campylobacter* or *Salmonella* (Stern, 1994). The MCE was used to inoculate each broiler chick to provide a relatively uniform intestinal flora. All day-of-hatch chicks were individually gavaged per os with 200 μL of MCE.

In each of 2 separate trials, day-of-hatch chicks were obtained from a local commercial hatchery and randomly allocated to 1 of 5 treatment pens/group: 1) negative controls, 2) challenge with 10^5 cfu of *Salmonella* spp. together with 10^4 cfu of *C. jejuni* per chick, 3) challenge with 10^6 cfu of *Salmonella* spp. together with 10^5 cfu of *C. jejuni* per chick, 4) challenge with 10^7 cfu of *Salmonella* spp. together with 10^6 cfu of *C. jejuni* per chick, or 5) challenge with 10^8 cfu of *Salmonella* spp. together with 10^7 cfu of *C. jejuni* per chick (n = 40/pen). Each treatment group was housed in an isolated floor pen (biosafety level class 2) with fresh pine litter, and the birds were provided water and feed ad libitum. In both trials, at d 4 posthatch, and at 1, 2, 3, and 4 wk posthatch, 8 birds from each of the 5 treatment pens (n = 40/sample day) were killed and ceca were aseptically collected for enumeration of *Salmonella* spp. and *C. jejuni* per gram of cecal materials.

Wk 4 Challenges

A total of 120 broiler chickens were used in this portion of the study. In each of 2 separate trials, day-of-hatch chicks were obtained from a local commercial hatchery and randomly allocated to 1 of 5 treatment groups. Each day-of-hatch chick was initially provided 200 μL of MCE by oral gavage. At wk 4 posthatch, 5 groups of birds were treated as follows: 1) negative control—no treatment, 2) each bird gavaged with 10 mg of vancomycin/0.5 mL, 3) each bird gavaged with 15 mg of vancomycin/0.5 mL, 4) each bird gavaged with 20 mg of vancomycin/0.5 mL, and 5) each bird gavaged with 25 mg of vancomycin/0.5 mL. Three hours later, the birds in treatment pens 2 through 5 were challenged via gavage with 200-μL suspensions of the composite 10^8-cfu *Salmonella* spp. suspension, described above. Each treatment group was housed in an isolated floor pen (Biosafety Level class 2) on fresh pine litter and provided water and feed ad libitum. In each trial, at wk 5 and 6 posthatch, 6 birds from each of the 5 treatment pens (n = 30/sample day) were killed, and individual ceca were collected for enumeration of *Salmonella* spp per gram of cecal materials.

Enumeration of C. jejuni and Salmonella spp. in Cecal Contents

The cecal contents of each broiler were initially suspended 1:3 and subsequently serially diluted 1:9 in PBS, and 100 μL of each dilution was plated onto Campy-Cefex agar plates (Stern et al., 1992). The plates were incubated for 48 h at 42°C under a microaerobic environment. After incubation, characteristic colonies were confirmed as *Campylobacter* by using a commercial latex agglutination test kit (PanBio Inc., Columbia, MD). The detection limit for *Campylobacter* was 1×10^2 cfu g^{-1} of cecal contents. From the same cecal suspensions, 100 μL of each dilution was plated onto brilliant green sulfa agar + 200 ppm of nalidixic acid plates. The plates were incubated for 24 h at 37°C in an incubator. The detection limit for *Salmonella* spp. was 1×10^2 cfu g^{-1} of cecal contents.

Statistics

The direct *Salmonella* spp. and *C. jejuni* counts were converted to $\log_{10}$ cfu per gram of cecal contents. The F-test (ANOVA, SAS version 9.1, SAS Institute Inc., Cary, NC) was used to determine significant differences among the d 4 through wk 4 mean values of each organism in each trial. To evaluate such differences further, the Scheffé S method (SAS version 9.1, SAS Institute Inc.) was applied to provide pair-wise comparisons between each data set. A *t*-test was used to compare the mean log values of the levels of organisms at wk 5 and 6.

Table 1. Colonization of chicks challenged at 2 d posthatch with a composite of 3 serotypes of *Salmonella* spp.[1]

Challenge level	d 4	wk 1	wk 2	wk 3	wk 4
Trial 1					
Negative control	ND[2]	ND	ND	ND	ND
10^5 cfu	5.46 ± 1.42	4.64 ± 0.91	2.80 ± 0.79	0.61 ± 0.91	ND
10^6 cfu	6.03 ± 1.06	6.02 ± 0.20	4.95 ± 1.24	3.01 ± 1.88	0.50 ± 0.70
10^7 cfu	5.34 ± 0.69	5.50 ± 0.71	1.79 ± 0.98	1.09 ± 1.00	ND
10^8 cfu	5.56 ± 1.41	5.14 ± 0.27	3.03 ± 1.39	1.47 ± 1.36	1.47 ± 1.68
Trial 2					
Negative control	ND	ND	ND	ND	ND
10^4 cfu	4.92 ± 1.21	4.97 ± 0.78	4.80 ± 1.67	5.34 ± 0.68	2.09 ± 1.93
10^5 cfu	6.68 ± 0.58	6.62 ± 0.40	5.89 ± 0.26	4.97 ± 0.51	2.47 ± 1.42
10^6 cfu	5.35 ± 0.84	5.85 ± 1.14	4.35 ± 1.57	3.56 ± 0.54	1.90 ± 2.18
10^7 cfu	6.02 ± 0.36	5.34 ± 1.02	4.46 ± 1.35	5.02 ± 1.15	2.17 ± 1.92

[1]Birds were grown for 4 wk. Colonization quotient expressed as $\log_{10}$ cfu g^{-1} of cecal materials of 8 individuals.

[2]ND = not detected.

RESULTS

The goal of this study was to produce a mature broiler chicken colonization model of *Salmonella* spp. and *C. jejuni*. Table 1 provides data to indicate that chicks given normal flora and subsequently challenged with a composite of 3 strains of *Salmonella* spp. were substantially colonized at 4 d posthatch. The least level of colonization was 8.3×10^4 cfu g^{-1} of cecal material, whereas the greatest level was 4.8×10^6 cfu g^{-1}. The increase in challenge level per chick did not provide a predictable arithmetic or logarithmic increase in colonization by *Salmonella* spp. In each of the 2 trials among all of the groups, colonization remained relatively stationary from d 4 to 7. In each experimental pen, colonization began to diminish by wk 2, and by wk 4, levels of *Salmonella* were either nondetectable or greatly diminished. Reductions in colonization ranged from 3 to 5 $\log_{10}$ g^{-1} of cecal material.

A very different picture was created with the *C. jejuni* chick challenge. Table 2 provides data to indicate that young chicks provided normal flora and subsequently challenged with a composite of 2 *C. jejuni* isolates were substantially colonized at numbers generally exceeding those found for *Salmonella* spp. From the first sample day (d 4) through wk 4, there was no reduction in *C. jejuni* colonization numbers, and the numbers generally increased with time. No increase in colonization was noted with increasing challenge levels.

Various levels of vancomycin were administered to 4-wk-old birds. Three hours after the antibiotic was administered and the gut flora were presumably disrupted, large quantities of *Salmonella* spp. (10^8 cfu $bird^{-1}$) were used as a challenge. One week later, colonization was monitored. Levels of resulting colonization ranged from 10^6 to 10^7 cfu/g of cecal material (Table 3). No apparent relation to the *Salmonella* numbers and the amount of vancomycin given to the birds was noted. At wk 6, colonization was reduced by approximately 3 $\log_{10}$, but the organism could still be enumerated among all the challenged birds.

DISCUSSION

Public exposure to poultry carcass-borne *Salmonella* occurs irrespective of whether the birds were colonized at the time of slaughter or whether the birds were colonized at the hatchery. *Salmonella* are known to be relatively durable bacteria and can survive comparatively well under dry conditions on the skin of birds. Contamination may occur when broilers rest on their keels. Hence, the carcasses of birds that roost on *Salmonella*-contaminated fecal materials can become contaminated at either an early or a mature phase of

Table 2. Colonization of chicks challenged at 2 d posthatch with a composite of 2 isolates of *Campylobacter jejuni*[1]

Challenge level	d 4	wk 1	wk 2	wk 3	wk 4
Trial 1					
Negative control	ND[2]	ND	ND	ND	ND
10^4 cfu	6.53 ± 0.65	7.54 ± 0.49	7.64 ± 0.48	7.29 ± 0.22	7.31 ± 0.41
10^5 cfu	5.48 ± 1.36	7.41 ± 0.53	7.83 ± 0.29	7.40 ± 0.22	7.63 ± 0.27
10^6 cfu	6.56 ± 0.41	6.95 ± 0.88	7.89 ± 0.28	6.88 ± 0.66	6.82 ± 0.70
10^7 cfu	7.20 ± 0.70	7.37 ± 1.50	7.60 ± 0.48	7.10 ± 0.88	6.55 ± 0.61
Trial 2					
Negative control	ND	ND	ND	ND	ND
10^4 cfu	6.36 ± 1.16	7.08 ± 0.53	7.22 ± 0.61	7.02 ± 0.51	7.83 ± 0.16
10^5 cfu	7.86 ± 0.65	7.41 ± 0.63	7.24 ± 0.47	7.63 ± 0.34	7.60 ± 0.79
10^6 cfu	5.97 ± 0.70	6.72 ± 0.16	7.95 ± 0.29	8.54 ± 0.35	8.27 ± 0.38
10^7 cfu	7.62 ± 0.94	7.09 ± 1.00	7.63 ± 0.26	7.50 ± 0.33	7.54 ± 0.34

[1]Birds were grown through 4 wk. Colonization quotient expressed as $\log_{10}$ cfu g^{-1} of cecal materials of 8 individuals.

[2]ND = not detected.

growth. However, the environmental sources (feed, water, insects, vermin, etc.) that bring *Salmonella* into a broiler house at some point in the production cycle can and do serve to propagate large numbers after colonizing the flock. It is thought that these large numbers are readily transferred to coopmates during transport of the birds to the processing plant. This is certainly the case with *Campylobacter* contamination of carcasses, because the pathogen levels on the bird carcasses increased 1,000-fold during cooping and transport (Stern et al., 1995). Hence, a need remains to assess alternative approaches for intervention in the pathogen colonization of the mature broiler and subsequent pathogen transfer onto the carcass.

The initial colonization of the day-of-hatch chicks with the MCE culture provided a wide array of organisms required for a consistent and healthy gut function. This was a similar approach that provided cecal subcultures from specific-pathogen-free birds for a healthy microflora before *Salmonella* challenge (Barrow et al., 2003). Challenging the birds with 10^4 cfu or more of *Salmonella* resulted in substantial (10^5 to 10^6 cfu g^{-1} of cecal material) gut colonization 4 d later. The large challenge numbers provided may be far greater than those normally occurring under commercial settings. Ten thousand-fold increases in challenge levels did not result in substantially greater colonization in either trial (Table 1). After 4 wk of colonization, *Salmonella* fell to nondetectable or nearly nondetectable levels. This observation was different from a previous study, which suggested that 10^8- to 10^9-cfu *Salmonella* challenges resulted in stable and lasting infections (Bjerrum et al., 2003). In the present study, a 6-wk-old broiler colonization model was not achieved by challenging chicks 2 d posthatch.

As demonstrated in numerous studies, once *Campylobacter* levels are established, they tend to remain at high levels throughout the life of the broiler. This certainly held true in our study, in which numbers were stable at 10^6 to 10^8 cfu g^{-1} of cecal material throughout the duration of the study (Table 2). The presence of the MCE did not appear to influence the colonization capacity of *Campylobacter*, and levels remained consistently high.

A consistent and high level of *Salmonella* colonization of the broiler gastrointestinal tract was achieved only by pretreating birds 4 wk posthatch with vancomycin. The level of prechallenge vancomycin did not appear to alter the counts of *Salmonella* in the gut. One week after challenge, the birds were consistently colonized with greater than 10^6 to 10^7 cfu g^{-1} of cecal material. At 6 wk posthatch, colonization dropped to a still countable $10^{2.4}$ to $10^{4.5}$ cfu g^{-1} of cecal material. It is likely that, as with the younger bird study, the intestinal flora were outcompeting the *Salmonella* and causing their numbers to diminish. It is suggested that a useable mature broiler *Salmonella* colonization model has been created, which appears suitable to study live bird interventions.

Table 3. Colonization of chicks treated with vancomycin at 4 wk of age and 3 h later challenged with 10^8 cfu $chicken^{-1}$ of a 3-isolate composite of *Salmonella* spp.[1]

Vancomycin level	wk 5	wk 6
Trial 1		
Negative control	ND[2]	ND
10 mg/bird	6.11 ± 1.27	2.38 ± 0.43
15 mg/bird	6.27 ± 1.04	3.66 ± 1.49
20 mg/bird	6.90 ± 0.48	3.56 ± 1.06
25 mg/bird	5.86 ± 0.72	2.85 ± 0.72
Trial 2		
Negative control	ND	ND
10 mg/bird	7.26 ± 0.36	3.52 ± 0.61
15 mg/bird	7.37 ± 1.02	3.14 ± 1.04
20 mg/bird	7.59 ± 0.47	3.60 ± 0.84
25 mg/bird	7.18 ± 1.79	4.47 ± 1.79

[1]Birds were grown through 6 wk. Colonization quotient expressed as $\log_{10}$ cfu g^{-1} of cecal materials of 8 individuals.

[1]ND = not detected.

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